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Zebrafish *topped* is required for ventral motor axon guidance

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Abstract

Zebrafish primary motor axons extend along stereotyped pathways innervating distinct regions of the developing myotome. During development, these axons make stereotyped projections to ventral and dorsal myotome regions. Caudal primary motoneurons, CaPs, pioneer axon outgrowth along ventral myotomes; whereas, middle primary motoneurons, MiPs, extend axons along dorsal myotomes. Although the development and axon outgrowth of these motoneurons has been characterized, cues that determine whether axons will grow dorsally or ventrally have not been identified. The *topped* mutant was previously isolated in a genetic screen designed to uncover mutations that disrupt primary motor axon guidance. CaP axons in *topped* mutants fail to enter the ventral myotome at the proper time, stalling at the nascent horizontal myoseptum, which demarcates dorsal from ventral axial muscle. Later developing secondary motor nerves are also delayed in entering the ventral myotome whereas all other axons examined, including dorsally projecting MiP motor axons, are unaffected in *topped* mutants. Genetic mosaic analysis indicates that Topped function is non-cell autonomous for motoneurons, and when wild-type cells are transplanted into *topped* mutant embryos, ventromedial fast muscle are the only cell type able to rescue the CaP axon defect. These data suggest that Topped functions in the ventromedial fast muscle and is essential for motor axon outgrowth into the ventral myotome.

Keywords: Motoneurons; Genetic mosaics; Genetic interactions; Axial muscle; Axon guidance; Intermediate targets

Introduction

During vertebrate nervous system development, motoneurons extend their axons in a highly stereotyped manner in order to reach their appropriate target muscles. The specificity of motor axon outgrowth is accomplished by growth cones as they integrate attractive and repulsive environmental cues. In the process of chemoattraction, diffusible/secreted or membrane-bound molecules present along axon pathways guide growth cones to both intermediate and distal targets. Identifying the cues critical for formation of motor nerves is essential for understanding neuromuscular development and diseases that may affect this process.

Motor axon pathfinding has been studied extensively in vertebrate limb, and differentially expressed dorsal or ventral cues appear essential for proper motor axon innervation. Studies in mouse revealed that *netrin1* and *sema3a* specifically repel dorsally extending axons (Varela-Echavarria et al., 1997). Motor axons in *EphA4* mutant mice can pathfind correctly to the plexus, but presumptive dorsal motor axons fail to extend into the dorsal limb and join the ventral nerve (Helmbacher et al., 2000). Overexpression of EphA4 also reveals dorsal nerve defects (Eberhart et al., 2002). Whereas there is some information about the cues involved in vertebrate limb innervation, almost nothing is known about the cues guiding motor axons to dorsal or ventral axial (body wall) musculature. In chick and mouse, motoneurons from the medial motor column innervate epaxial (dorsal) and hypaxial (ventral) body wall muscle. The target epaxial muscle derived from the dermamyotome is essential for epaxial nerves to form

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suggesting that guidance or attractive cues are present in the muscle (Tosney, 1987). However, differential cues important for dorsal or ventral axial muscle innervation in amniotes have not been described.

Zebrafish is an excellent model organism to study axial muscle innervation since the vast majority of the motoneurons innervate these muscles with only a minority of motoneurons innervating the pectoral fins, the evolutionary equivalent of the forelimb. Moreover, there is an entire subset of early developing primary motoneurons that only innervates axial muscle. Each of the three primary motoneurons is uniquely identified by its soma position within the spinal cord, characteristic gene expression, and stereotyped axonal projection (for reviews, see Beattie, 2000; Eisen, 1999). Caudal primary (CaP) motoneurons innervate ventral axial muscle, middle primary (MiP) motoneurons innervate the dorsal axial muscle, and rostral primary (RoP) motoneurons innervate the middle muscle territory. The CaP motoneuron has been studied quite extensively because its soma, in the middle of each spinal cord hemisegment, is relatively easy to visualize and it has a prominent ventral axon projection. At approximately 18 h postfertilization (hpf) in each spinal cord hemisegment, the CaP growth cone leaves the spinal cord and extends ventrally until it reaches the first intermediate target, the nascent horizontal myoseptum (often called the choice point), which demarcates dorsal from ventral muscle. Intermediate targets are regions along an axon pathway where growth cones pause, branch, or turn implying that information is being imparted. After pausing for 1–2 h, the CaP axon continues past the first intermediate target along the ventral myotome next to the notochord (Fig. 1; Eisen et al., 1986; Myers et al., 1986). In avian embryos, the sclerotome is immediately adjacent to the notochord and motor axons extend along anterior sclerotome (Keyes and Stern, 1984). In zebrafish, however, myotome is immediately adjacent to the notochord (see Morin-Kensicki and Eisen, 1997). Ablation of the ventromedial cell cluster, which contains all the cells that will give rise to the sclerotome in addition to some other cells types including muscle cells, resulted in a mild CaP axon defect, a stunted appearance at 27–30 hpf, that subsequently recovered (Morin-Kensicki and Eisen, 1997). The authors concluded that unlike in avians, sclerotome does not play a central role in zebrafish motor axon outgrowth and suggested that myotome may function in this capacity. They go on to suggest that the transient stunted appearance of CaP may have been due to removal of muscle cells that derive from the ventromedial cell cluster. Motor axon outgrowth along the ventromedial fast muscle implies the presence of ventrally located guidance cues, but none have yet been identified.

Mutagenesis screens in zebrafish have begun to uncover mechanisms of axial muscle innervation. The gene *stumpy* appears to function as a cue needed for motor axons to proceed past intermediate targets (Beattie et al., 2000).

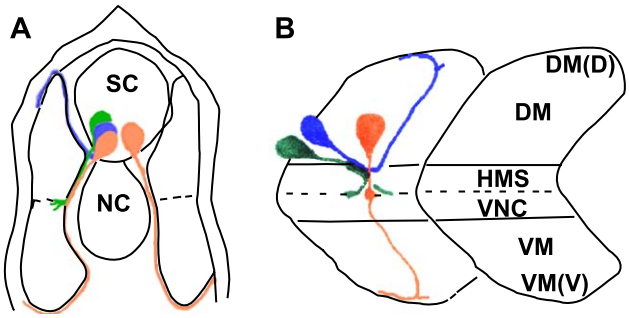


Fig. 1. Schematic diagram of the primary motor axon pathways at 26 hpf. (A) Cross-sectional view of the three primary motoneurons. All three primary motoneurons are shown on the left and only CaP is shown on the right; CaP (orange), MiP (blue), and RoP (green). (B) Lateral view of spinal cord hemisegments with the primary motoneuron cell bodies shown in their stereotyped positions in the ventral spinal cord. CaP growth cones exit the spinal cord at approximately 18 hpf and extend ventrally until they reach the first intermediate target, the nascent horizontal myoseptum (dashed line), where they pause for 1–2 h. The CaP axon then continues ventrally along the ventromedial myotome. MiP axons extend along the dorsomedial myotome, and RoP axons extend through the horizontal myoseptum. Due to the normal variability in axon outgrowth at 26 hpf, schematics are representative. The hemisegment on the right indicates myotome regions that will be used for scoring growth cone position. DM(D), dorsal-most region of the dorsal myotome; DM, dorsal myotome; HMS, horizontal myoseptum; VNC, myotome adjacent to the ventral edge of the notochord; VM, ventral myotome; VM(V), ventral-most region of the ventral myotome. sc, spinal cord; nc, notochord.

Granato et al. have identified two cues functioning in slow muscle, *diwanka* and *unplugged*, that are essential for establishing the common pathway shared by all of the primary motor axons and for CaP and RoP to make the correct pathway choices, respectively (Zeller and Granato, 1999; Zhang and Granato, 2000). However, cues have not yet been identified that define axon pathways on the ventral and/or dorsal myotome. Here we characterize the *topped* mutant phenotype. We find that in *topped* mutants, motor axon outgrowth into the ventral myotome is specifically affected. Based on rescue experiments, we reveal that Topped is acting in ventromedial fast muscle to promote ventral axon outgrowth. Our data support the idea of differential dorsal or ventral cues dictating stereotyped motor axon pathways along axial muscle.

Materials and methods

Fish strains and maintenance

Mutant strains were maintained as heterozygous (*topped*^{b458}) lines in the Tuebingen long fin (TL)/AB* background. Homozygous mutant *topped* embryos were generated by pairwise mating of *topped* heterozygous fish or mating homozygous adults. Embryos raised from matings were maintained between 25.5 and 28.5°C and staged by converting the number of somites to hours postfertilization (hpf; Kimmel et al., 1995).

Generation of mutants

A mutagenesis screen was conducted (Beattie et al., 1999). Briefly, after exposure to 3 mM ethylnitrosourea (ENU) (Solnica-Krezel et al., 1994), adult male fish were outcrossed to wild-type females to create the F_1 generation. F_1 females were screened for mutations using the early pressure method to generate parthenogenetic diploid F_2 embryos (Streisinger et al., 1981). The embryos were fixed and labeled with antibodies to identify mutants. F_1 females carrying mutations of interest were outcrossed and lines generated.

Genetic mapping

To map *topped*, mutants in the *AB background were outcrossed to the TL strain to create a polymorphic mapping line. Parthenogenetic F_2 diploid embryos were produced by fertilizing eggs from heterozygous females with UV-irradiated sperm (Streisinger et al., 1981). Individual embryos were scored as mutant or wild type based on antibody labeling. Genomic DNA was isolated and PCR was performed with simple sequence length polymorphic markers to obtain centromeric linkage (Johnson et al., 1995, 1996).

Whole-mount antibody labeling

Whole-mount antibody labeling was performed as described (Beattie et al., 2000; Eisen et al., 1989). The znp1 monoclonal antibody that recognizes primary and secondary motor axons (1:100; Melancon et al., 1997; Trevarrow et al., 1990), anti-acetylated tubulin (1:500; Zymed Laboratories, Inc.), 4D9 (1:50; Devoto et al., 1996; Patel et al., 1989), and 3A10 (1:10; Hatta, 1992) were detected using the Sternberger Clonal-PAP system with diaminobenzidine (DAB) as a substrate (Beattie and Eisen, 1997) or with Oregon Green® goat anti-mouse IgG (Molecular Probes). Embryos were analyzed with a Zeiss Axioplan microscope, and images were captured with Kodak Ektachrome 64Y film or digitally imaged using a BioRad (MRC 1024) confocal microscope. 4D9, developed by C. Goodman, and 3A10, developed by T. Jessell and J. Dodd, were obtained under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa Department of Biological Sciences.

Whole-mount RNA in situ hybridization

Whole-mount RNA in situ hybridization was performed as described by Thisse et al. (1993). An antisense digoxigenin *myoD* riboprobe was synthesized from a plasmid linearized with *Xba*I and transcribed with T7 (Weinberg et al., 1996). A *robo1* riboprobe was synthesized from the 5′end of a *robo1* clone (Challa et al., 2001). The *crestin* riboprobes were synthesized from a plasmid linearized with *Eco*R1 and transcribed with T7 (Rubinstein et al., 2000).

Single cell labeling

Individual motoneurons were iontophoretically labeled with rhodamine dextran (3×10^3 MW; Molecular Probes) as previously described (Beattie et al., 2000; Eisen et al., 1989; Westerfield, 1995). Labeled cells were visualized with a Zeiss Axioskop. Images were captured with a Photometrics SPOT camera and were colorized using Photoshop (Adobe).

Blastula transplants

To generate mosaic embryos, cells were transplanted between wild-type and mutant embryos as described (Ho and Kane, 1990). Donor embryos were injected with rhodamine dextran (3×10^3 MW; Molecular Probes) at the 2-cell stage. Transplantation of blastula cells was conducted from 3 to 3.7 hpf. Embryos were fixed at 26 hpf for 2 h at room temperature in 4% paraformaldehyde. *topped* mutant embryos were identified by immunohistochemistry with znp1 monoclonal antibody, and immunoreactivity was detected with Oregon Green® goat anti-mouse IgG (1:200; Molecular Probes). Transplanted cells were visualized using fluorescence and confocal microscopy. For cross-sectional analysis of rescued motor axons, embryos were embedded in 1.5% agar/5% sucrose and sectioned on a cryostat at 16 μm. Sections were then processed for immunohistochemistry with znp1 and F310 monoclonal antibodies (Crow and Stockdale, 1986), and immunoreactivity was detected with isotype-specific conjugate secondary antibodies Alexa Fluor® 350 IgG1 (F310) and Alexa Fluor® 488 IgG2a (znp1) (1:300; Molecular Probes). Sections were analyzed with a Zeiss Axioskop and images were captured with a Photometrics SPOT camera.

Genotyping from blastulae

Embryos were collected from *topped* heterozygous matings and allowed to develop to 3 hpf. The embryos were then mounted in 4% methyl cellulose in an agar mold. The tray was flooded with embryo medium containing 100 units penicillin and 100 μg streptomycin/ml. Approximately 10 cells were removed using a 10-μl electrode (VWR) with a 40-μm diameter opening using a standard blastula transplant apparatus (Ho and Kane, 1990). The cells were then transferred to a sterile staining dish containing 5 μl of a 400 ng/μl ProK/17 μM SDS solution (adapted from Troeger et al., 1999). The solution containing the cells was then transferred to PCR tubes and incubated at 50°C for 1 h and subsequently denatured at 99°C for 30 min. To each tube, 25 μl of PCR mix containing dNTPs, $MgCl_2$, and 25 pM of the closely linked marker z58867 was added and amplified. Mutants were identified as those that segregated with z58867.

Results

topped is a recessive, semi-lethal mutation

In a screen designed to elucidate genes that affect motor axon pathfinding, the *topped*^{b458} (*topped*) mutation was identified (Beattie et al., 1999, 2000). *topped* is an ethlynitrosourea-induced, autosomal recessive mutation that displays Mendelian inheritance. There is approximately 70% homozygous viability, while the remaining 30% die around 14 days postfertilization (dpf). As a first step towards identifying the molecular nature of the *topped* gene, we placed it onto the genetic map of the zebrafish genome. We used simple sequence length polymorphic markers on early parthenogenetic diploid embryos (EP; Johnson et al., 1995, 1996) to map *topped* to a chromosome. Marker z3399 on chromosome 24 segregated with *topped* in all mutants examined (n = 50 EP diploid embryos) indicating that *topped* maps to this chromosome. Fine mapping with haploid *topped* mutant embryos as well as the ratio of mutant:wild-type EP diploid embryos (data not shown) indicate that *topped* is close to the centromere.

CaP axons in topped mutants are delayed entering the ventral myotome

Antibody labeling with the znp1 monoclonal antibody revealed that CaP axons in *topped* mutants stalled at or near the first intermediate target, the nascent horizontal myoseptum (Fig. 2). This phenotype was penetrant but exhibited some variable expressivity. For example, some F_1 heterozygous adults produced mutant embryos in which approximately 74% of CaP axons stalled at the first intermediate target at 26 hpf (100 axons scored in 10 fish); the remainder extended slightly beyond this region (Fig. 2C). However, even in these mutants, CaP axons never extended into the distal ventral myotome as is seen in wild-type embryos. Other F_1 heterozygous adults yielded mutants in which 98% of CaP axons stalled at the first intermediate target at 26 hpf (232 axons scored in 43 fish), while the remaining 2% were near the ventral edge of the notochord. F_1's that produced embryos exhibiting the more severe mutant phenotype were used for all subsequent experiments. For consistency, only axons in hemisegments 6–12 were analyzed.

To analyze the CaP axon defect in more detail, we labeled individual CaP somata in live *topped* mutant embryos and visualized their axon projections over time. Since *topped* mutants have no visible morphological phenotype and can only be identified by their CaP axon phenotype, we extracted 10–20 cells from blastula stage embryos from heterozygous matings and genotyped them using closely linked markers to identity live mutants. These embryos developed properly and at approximately 22 hpf we labeled single CaP motoneurons iontophoretically with a vital fluorescent dye in embryos that were genotypically wild type or mutant (Fig. 3; also see Table 1). Once we established a *topped* homozygous mutant line, this method was not necessary. In wild-type embryos at 22 hpf, CaP growth cones had already extended past the first intermediate target (data not shown); and by 25–26 hpf, wild-type CaP growth cones had extended into the ventral myotome (Fig. 3A). In *topped* mutants, however, CaP growth cones were still stalled at the first intermediate target at 25–26 hpf (25/27; Fig. 3D). By 30 hpf, however, CaP growth cones in *topped* mutants had extended past the first intermediate target into the ventral myotome (23/23; Fig. 3E). Interestingly, the axons did not stall at the second putative intermediate target, the myotome adjacent to the ventral edge of the notochord, a phenotype seen in *stumpy* mutants (Beattie et al., 2000). This suggests that the CaP axon stall phenotype in *topped* mutants is not due to defects at all intermediate targets. By 45 hpf, CaP axons in *topped* mutants looked indistinguishable from wild-type CaP axons (18/18; Fig. 3F). These data indicate that *topped* is important for CaP axons to extend into the ventral myotome. The finding that CaP axons eventually recover suggests either that we do not have a null allele or that more than one gene is involved in this process.

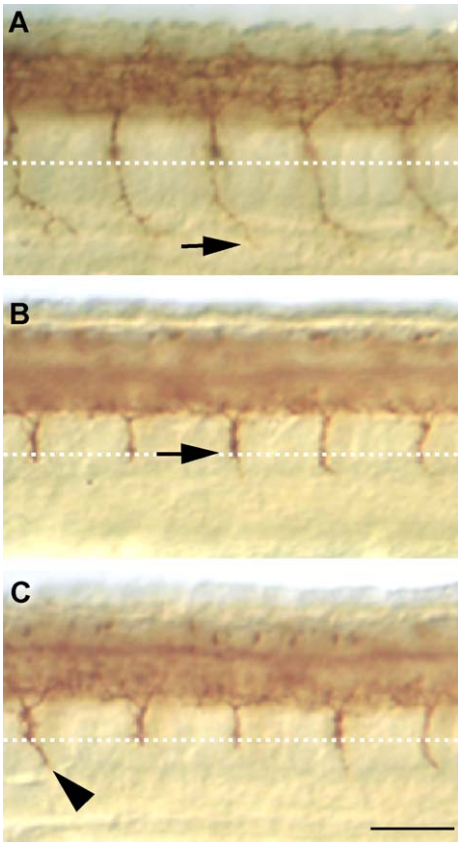


Fig. 2. CaP axons in *topped* mutants exhibit a stall phenotype at the first intermediate target. Lateral views of whole-mount antibody labeling with znp1 of 26 hpf *topped* wild-type sibling (A) strong homozygous (B) and weak homozygous (C) mutants. The dashed line denotes the first intermediate target. Arrows indicate CaP growth cone position. Arrowhead indicates a CaP axon extending past the first intermediate target. For this and all subsequent lateral views, dorsal is to the top and anterior is to the left. Scale bar, 35 μm.

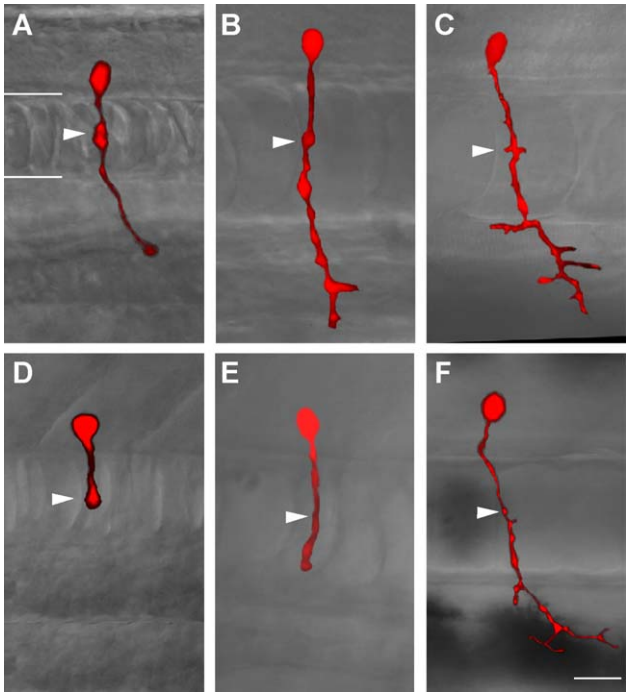


Fig. 3. CaP axons are delayed entering the ventral myotome in *topped* mutants. Individual CaP cell bodies were iontophoretically labeled in living wild-type (A–C) and *topped* mutants (D–F) with rhodamine dextran and imaged at approximately 25 hpf (A and D), 29 hpf (B and E), and 45 hpf (C and F). Axons were imaged along the medial pathway. Arrowheads denote the first intermediate target. White lines indicate the dorsal and ventral aspects of the notochord. Scale bar, 25 μm.

Topped is required for the proper outgrowth of ventral motor nerves

A second population of later developing motor axons also extends into the ventral myotome. There are approximately 20–30 secondary motoneurons whose axons fasciculate together with the CaP axon to form the ventral nerve (Myers, 1985; Myers et al., 1986; Pike et al., 1992). Secondary motor axons begin to extend at approximately 26 hpf and continue to emerge until approximately 34 hpf (Myers et al., 1986). Analysis of *topped* mutants at 29–35 hpf revealed that ventral nerve outgrowth was delayed. In wild-type embryos at 30 hpf, approximately 15 secondary motor axons contribute to the ventral nerve and extend to the most ventral aspect of the myotome (Pike et al., 1992). In *topped* mutants at 30 hpf, however, ventral nerves were aberrant with approximately 6% still at the first intermediate target (Fig. 4B, arrowhead) and approximately 52% stalled at the myotome adjacent to the ventral edge of the notochord (Fig. 4B, arrow). The remaining nerves extended further ventrally, but only 8% of the nerves reached the ventral-most aspect of the myotome. This is in contrast to CaP growth cones in *topped* mutants at 29 hpf, which are just past the first intermediate target as revealed by single cell labeling (see Fig. 3E). By 34 hpf, 98% the ventral nerves had recovered and reached the ventral-most aspect of the myotome. Thus, both populations of ventrally extending motor axons, primaries and secondaries, show delays in entering the ventral myotome in *topped* mutants.

Dorsally projecting axons are unaffected in topped mutants

The finding that growth cones were stalling at the first intermediate target and were delayed entering the ventral myotome suggested that *topped* might function as a ventral motor axon guidance cue. If this were the case, we would predict that dorsally projecting MiP axons would be unaffected in *topped* mutants. To obtain a detailed analysis of MiP axons, we injected somata of MiP motoneurons with a vital fluorescent dye and followed their projections in living *topped* mutant embryos (Fig. 5; Table 1). We found that MiP axons in *topped* mutants were identical to wild-type MiP axons. At the first time point analyzed, 26 hpf, MiP axons had extended dorsally in both wild-type and *topped* mutant embryos (Table 1). By 29 hpf, MiPs in *topped* mutant embryos had proceeded into the distal dorsal myotome (8/8; Fig. 5B), and in one case the axon had already turned to extend along the myotome boundary consistent with what is

Table 1
MiP axons in *topped* mutants exhibit no delays in outgrowth onto the dorsal myotome

Fish	Genotype	Motoneuron	Segment	26 hpf	29 hpf	45 hpf
1	*topped*$^{-/-}$	MiP	6	dm	dm(d)	dm(d)
		CaP	5	hm	vnc	*
2	*topped*$^{-/-}$	MiP	6	dm	dm(d)	dm(d)
		CaP	4	hm	vnc	*
3	*topped*$^{-/-}$	MiP	7	dm	dm(d)	dm(d)
		CaP	5	hm	vnc	*
4	*topped*$^{-/-}$	MiP	7	dm	dm(d)	dm(d)
		CaP	10	hm	vnc	vm(v)
5	*topped*$^{-/-}$	MiP	9	dm	dm(d)	dm(d)
		CaP	7	hm	vnc	vm(v)
6	*topped*$^{-/-}$	MiP	6	dm	dm(d)	dm(d)
		CaP	6	hm	vnc	vm(v)
7	*topped*$^{-/-}$	MiP	6	dm	dm(d)	dm(d)
		CaP	6	hm	vnc	vm(v)
8	*topped*$^{-/-}$	MiP	8	dm	dm(d)	dm(d)
		CaP	7	hm	vnc	vm(v)
9	Wild type	MiP	7	dm	dm(d)	dm(d)
		CaP	9	vm(v)	vm(v)	vm(v)
10	Wild type	MiP	6	dm	dm(d)	dm(d)
		CaP	5	vm(v)	vm(v)	vm(v)
11	Wild type	MiP	6	dm	dm(d)	dm(d)
		CaP	8	vm(v)	vm(v)	*
12	Wild type	MiP	7	dm	dm(d)	dm(d)
		CaP	6	vm(v)	vm(v)	vm(v)
13	Wild type	MiP	6	dm	dm(d)	dm(d)
		CaP	5	vm(v)	vm(v)	vm(v)
14	Wild type	MiP	8	dm	dm(d)	dm(d)
		CaP	7	vm(v)	vm(v)	vm(v)

Individual MiP and CaP cell bodies were labeled in the same fish with vital fluorescent rhodamine dextran and growth cone position was scored (see Fig. 1B for abbreviations) at 26, 29, and 45 hpf. Note: Only CaPs in fish 5–8 were included in the characterization of the CaP *topped* mutant phenotype. Asterisk (*) indicates time point not scored.

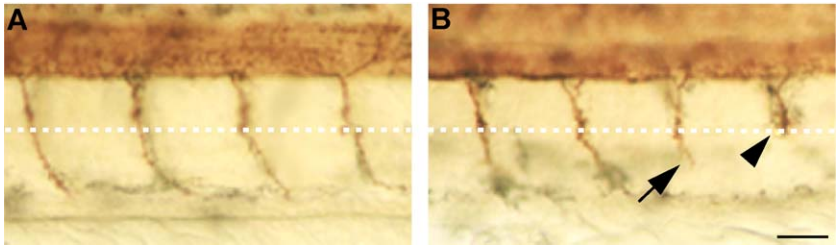


Fig. 4. Formation of ventral nerves is delayed in *topped* mutants. Lateral view of wild-type (A) and *topped* mutant (B) embryos labeled with znp1 at 30 hpf. The first intermediate target is denoted by the white dashed line. The arrowhead indicates a nerve stalled at the first intermediate target, and the arrow indicates a nerve stalled at the myotome adjacent to the ventral edge of the notochord. Scale bar, 20 μm.

seen in wild-type embryos (Eisen et al., 1986; Myers et al., 1986). At both of these time points, CaP axons in the same embryos were severely affected (Table 1) showing that while CaP axons are delayed in entering the ventral myotome in *topped* mutants, MiP axons experience no such delays in entering the dorsal myotome. By 45 hpf, all mutant MiP axons examined had made their stereotyped projection along the rostral myotome boundary. We also examined RoP axons, which branch and extend laterally at the first intermediate target, by intracellar dye labeling. While there was some variability in the timing of the appearance of the initial forked branch that forms around 25 hpf, by 30 hpf there was no difference between RoP axons in *topped* mutants and wild-type embryos (Figs. 5C–D). These data indicate that Topped function is only necessary for ventral motor axon guidance.

Other neuronal cell types and myotome are unaffected in topped mutants

To test the specificity of the *topped* mutation for ventral motor axons, we examined the projections and subsequent outgrowth of sensory and interneurons in *topped* mutants. The medial longitudinal fascicle and the lateral longitudinal fascicle contain hindbrain and spinal interneuron axons. We analyzed these large axon tracts with acetylated tubulin antibody at 18, 24, 30, and 36 hpf and saw no defects in their outgrowth in *topped* mutants. We also examined the hind-

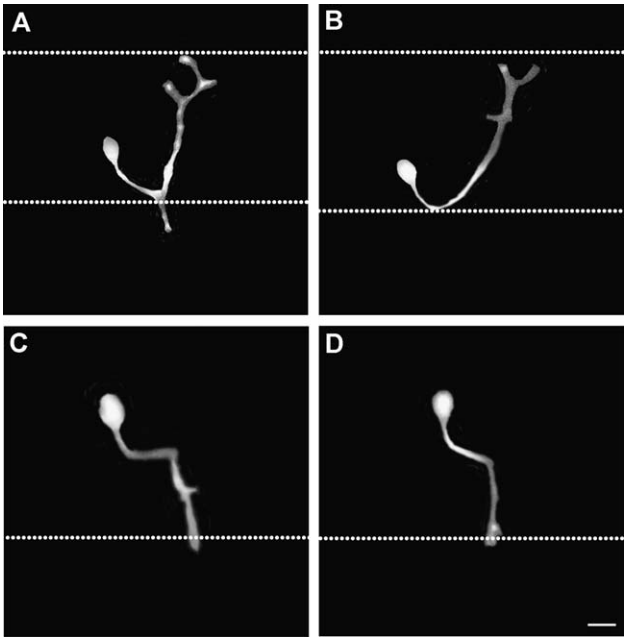


Fig. 5. MiP and RoP axons are unaffected in *topped* mutants. Iontophoretically labeled MiP motoneurons in living wild-type (n = 6; A) and *topped* mutants (n = 8; B) at 29 hpf. Dashed lines in A and B indicate the dorsal and ventral aspects of the spinal cord. RoP motoneurons were labeled in living wild-type (n = 5; C) and *topped* mutants (n = 5; D) and imaged at 30 hpf. Dashed line in C and D indicates the first intermediate target. Scale bar, 20 μm.

Table 2
Other axons are unaffected in *topped* mutants

	Age (hpf)	Probe	wt	*topped*$^{-/-}$
Mauthner	24	3A10 antibody	222.3 ± 0.1	222.3 ± 0.1
axons[a]	30		698.8 ± 0.1	701.3 ± 0.1
	36		20.4 ± 0.0[b]	20.3 ± 0.0
Trigeminal	24	anti-acetylated	155.2 ± 0.1	154.9 ± 0.1
ganglia	30	tubulin antibody	336.9 ± 0.2	333.4 ± 0.2
axons[c]	36		397.6 ± 0.2	399.9 ± 0.1
Lateral line	30	anti-acetylated t	6.9 ± 0.0	6.9 ± 0.0
axons[d]	36	ubulin antibody	17.8 ± 0.1	17.9 ± 0.0
Lateral	24	*robo1* riboprobe	4.9 ± 0.0	4.9 ± 0.0
line	28		10.8 ± 0.0	10.8 ± 0.0
primordium[e]	34		21.8 ± 0.0	21.6 ± 0.0

Axon lengths (a–d) and primordia migration (e) were analyzed at specific stages (age) using immunohistochemistry or RNA in situ hybridization (probe). Positions or lengths are reported as mean ± 95% confidence interval. Ten embryos were scored for each data point. No significant differences were found using a Student's t test between wild-type and *topped* mutant embryos ($P > 0.32$).

[a] Mauthner axon lengths were measured (μm) from cell bodies to growth cones at 24 and 30 hpf.

[b] At 36 hpf, Mauthner axon lengths were scored by growth cone hemisegment position.

[c] Trigeminal axons were measured (μm) as radial distance from the center of the ganglia.

[d] Lateral line axon lengths were scored by growth cone hemisegment position.

[e] Lateral line primordia migration was scored by hemisegment position.

brain Mauthner interneuron with 3A10 antibody at 24, 30, and 36 hpf and saw no phenotypic difference in wild-type and *topped* mutant embryos (Table 2; Supplemental Figs. 1A and B). The trigeminal ganglia (Table 2), Rohon–Beard sensory neurons that innervate the peripheral myotome and skin (Supplemental Figs. 1E and F), and the lateral line sensory axons that extend along the length of the embryo (Table 2; Supplemental Figs. 1C and D) were all examined at 18, 24, 30, and 36 hpf with acetylated tubulin antibody. All projections from these cells exhibited no delay in their outgrowth and appeared phenotypically wild type in *topped* mutants.

Since growth cone motility is mechanistically similar to cell motility (discussed in Giniger, 2002), we examined the possibility that *topped* is functioning globally in cell migration. Neural crest migration follows the same migratory pathway as primary motor axon growth cones. We examined neural crest migration at 16, 18, and 22 hpf by in situ hybridization with the *crestin* riboprobe that labels migrating neural crest cells (Luo et al., 2001; Rubinstein et al., 2000) in wild-type and *topped* mutant embryos. We saw no abnormalities in neural crest cell migration in *topped* mutants (data not shown) indicating that Topped does not function globally in cell motility.

To determine if the ventral motor axon defect in *topped* mutants was due to defects in muscle development or patterning, we examined the patterning and subsequent development of the myotome. Upon visual inspection using Normarski imaging, no difference in morphology was apparent in *topped* mutants compared to wild-type (data not shown). To determine if *topped* myotomes were correctly patterned, we performed in situ hybridization with a *myoD* riboprobe at 14, 16, and 18 hpf (Weinberg et al., 1996; Supplemental Figs. 2A and B). At all time points analyzed, *myoD* expression was identical in wild-type and *topped* mutants. We further examined the specification of the myotome by using separate markers for fast and slow muscle. Using the antibody 4D9 (Patel et al., 1989) that recognizes the engrailed protein in muscle pioneers, a subset of slow muscle (Supplemental Figs. 2C and D), and the antibody F310 (Crow and Stockdale, 1986) that recognizes fast muscle (see Fig. 8), we saw no difference in expression in wild-type and *topped* mutant embryos. Lastly, to insure an intact horizontal myoseptum, we examined the lateral line primordium using a *robo1* riboprobe at 24, 28, and 34 hpf (Challa et al., 2001; Table 2; Supplemental Figs. 2E and F). We saw no difference in the location or morphology of the primordium in *topped* mutants compared to wild-type embryos. These data suggest that muscle morphology and patterning are not disrupted in *topped* mutants.

Genetic mosaics reveal that Topped function is non-cell autonomous for CaP axons

Topped could be functioning in CaP axons or in the environment to enable axon outgrowth into the ventral myotome. To address this issue, we used blastula transplantation to create genetic mosaic embryos (Ho and Kane, 1990). We transplanted donor rhodamine-labeled cells into age-matched host embryos at 3.3 hpf (high stage). Cells were transplanted from wild-type donors into host embryos obtained from heterozygous *topped* matings. Alternatively, cells from donor embryos obtained from heterozygous *topped* matings were transplanted into wild-type host embryos. After fixation at 26 hpf, the identity of both donor and host was determined by immunohistochemistry with the znp1 antibody. In wild-type embryos, *topped* mutant CaP motoneurons had axons that exhibited wild-type outgrowth and morphology (Figs. 6A–C; Table 3). In the reciprocal experiment, wild-type CaP axons in *topped* mutant host embryos stalled at the first intermediate target; a phenotype consistent with *topped* mutants (Figs. 6D–F; Table 3). These data reveal that Topped function is needed in the environment and not in CaP motoneurons.

Topped is required in ventromedial fast muscle

Additional genetic mosaics were generated to determine the precise location of wild-type cells that rescued the *topped* mutant phenotype. For these experiments, blastula

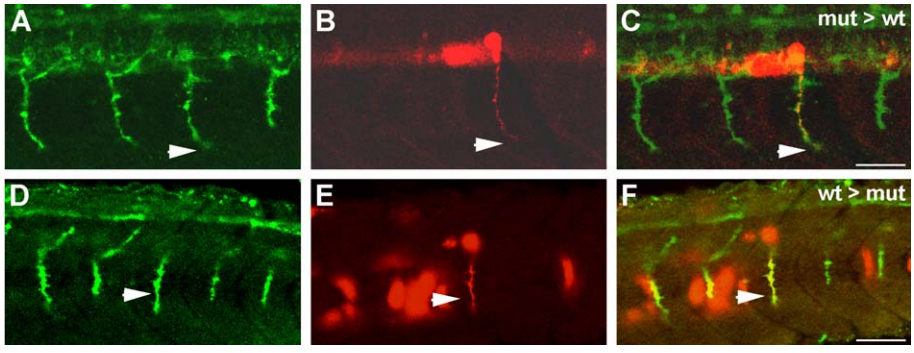


Fig. 6. Topped acts non-cell autonomously with respect to CaP. (A and D) FITC image of znp1-labeled CaP axons. (B and E) Transplanted rhodamine-labeled cells. (C and F) Merged images. (A–C) Lateral views of a 27 hpf wild-type host embryo containing transplanted cells derived from a rhodamine-labeled *topped* mutant donor. White arrows indicate a mutant CaP with a wild-type morphology. (D–E) Lateral view of a 27-hpf *topped* mutant embryo containing transplanted cells derived from a wild-type donor. White arrows indicate a wild-type CaP exhibiting a stall phenotype in a *topped* mutant. Scale bar, 40 μm.

Table 3
Topped is non-cell autonomous for CaP motoneurons

Donor genotype	Host genotype	Result
Wild type	wild type	14/14 wild type (100%)
Topped$^{-/-}$	wild type	12/12 wild type (100%)
Wild type	*topped*$^{-/-}$	12/12 mutant (100%)

Donor embryos were labeled with rhodamine dextran. Cells were transplanted from wild-type donors into embryos collected from a heterozygous *topped* mating and vice versa to obtain the above scenarios. Donor and host embryos were fixed at 26 hpf and antibody labeled to determine genotype. The embryos were then examined for the presence of rhodamine-labeled transplanted CaP motoneurons. The phenotype of the transplanted CaP axons (number and percentage) are reported.

stage cells were transplanted from wild-type donors into embryos obtained from *topped* heterozygous or homozygous matings. The resulting mosaic embryos were fixed at 26 hpf and stained with znp1 antibody to determine their genotype. Analysis of 281 mosaic *topped* mutant embryos in lateral whole mount revealed that notochord, floor plate, and lateral muscle cells were not able to rescue CaP axons (Table 4). However, in cases where wild-type medial muscle cells were present, CaP axons exhibited rescue (Table 4). Interestingly, the dorsoventral location of the transplanted muscle cells was also imperative. CaP axons recovered to the dorsoventral position of the ventral-most wild-type muscle cell (Figs. 7C and F). In addition, CaP axons appeared to be able to extend across two but not three or more mutant muscle cells to reach a wild-type muscle cell (Fig. 7C, compare axons 3 and 4). Although we often saw a correlation between rescued axons and wild-type transplanted muscle cells (Fig. 7C), there were cases in which muscle cells were located in the medial ventral myotome and failed to rescue CaP axons (Fig. 7F, axons 3 and 4). Additional analysis was necessary to resolve these examples.

To further examine the position and identity of wild-type clones that rescued CaP axons in *topped* mutants, we sectioned the mosaic *topped* mutant embryos and used muscle-type-specific antibodies (Fig. 8). Zebrafish myotome is composed of fast muscle derived from the lateral presomitic mesoderm and a single layer of lateral slow muscle derived from adaxial cells (Devoto et al., 1996). The F310 antibody was used to confirm the identity of fast muscle (Crow and Stockdale, 1986). Analysis of the rescued axons revealed that wild-type cells that rescued CaP axons in *topped* mutants were ventromedial fast muscle cells (Figs. 8A–H; Table 4). Interestingly, the cells had to be located at the most medial location of the fast myotome to rescue mutant CaP axons. To summarize this data, we generated a schematic of the fast muscle cells in the ventral myotome, indicating cell position by a number (dorsoventral axis) and letter (mediolateral axis; Fig. 9A). We found CaP axon rescue in 45 hemisegments; analyzing a subset of these, we found that in 19/19 cases, the rescuing cells were located in the A position (Fig. 9B). In 17/19 cases, the cell 1A was a wild-type transplanted cell, indicating that Topped function in this first ventromedial muscle cell is important for CaP axons to enter the ventral myotome. In the remaining two cases, the first rescuing cell was either 2A or 3A. However, in two other cases where cell 3A was the first wild-type cell present, we found no rescue; thus, cell 3A only rescued 33% of the time when present as the first transplanted muscle cell. In no cases did we see further ventral cells (e.g., 4A–6A) rescue when present as the first or only wild-type transplanted muscle cell. Furthermore, while we saw gaps of one or two cells still rescue (Fig. 9C, examples 3, 4, and 5), gaps of more than two cells never rescued mutant axons. These data indicate that two cells are the limit that wild-type cells, and thus Topped function, can act.

Wild-type muscle cells that failed to rescue support the hypothesis that Topped is functioning specifically in ventromedial fast muscle cells. We found that even though transplanted cells were located in row 1 or 2, they were not able to rescue in any letter position other than A. For example, we examined 12 cases of non-rescue where wild-type muscle cells were located in position 1B or 2B. This strict medial requirement for Topped function was supported by the example shown in Figs. 8E–H. Both hemisegments contained wild-type fast muscle cells in position 1A; however, the non-rescued side contained a wild-type cell in position 3B and the rescued side contained a wild-type cell in position 4A, suggesting that this medial A location is essential for wild-type cells to rescue. We were also able to resolve examples where we saw ventral muscle in lateral views but saw no rescue. We found in these cases that the muscle cells were not in the most medial A position. For example, we examined axons 3 and 4 from Fig. 7F in cross section and found that wild-type muscle cells were present in positions 2B, 2C, and 3B for axon 3 and cells 3B and 4B where present in the section with axon 4.

That other muscle cells did not rescue *topped*$^{-/-}$ CaP axons supports the idea that the location of Topped function is crucial for ventral motor axon outgrowth. We found that even large numbers of wild-type lateral fast muscle were unable to rescue CaP axons in *topped* mutants (Figs. 8I–L;

Table 4
Topped function is required in ventromedial fast muscle

Cell type	*n*	Percentage of rescue
Medial fast muscle	45 (25)	100
Lateral fast muscle	46 (23)	0
Slow muscle	17 (14)	0
Notochord	33 (15)	0
Floor plate	18 (10)	0

Wild-type cells were transplanted into *topped* mutant embryos and the ability of particular cell types to rescue CaP axons was assayed. To determine the type and position of transplanted muscle, a subset of the embryos were sectioned. Numbers for notochord and floor plate were counted only when the entire hemisegment contained transplanted cells (approximately three cells per hemisegment); *n* = the number of hemisegments where the various cell types were present and the number in parenthesis corresponds to the number of embryos.

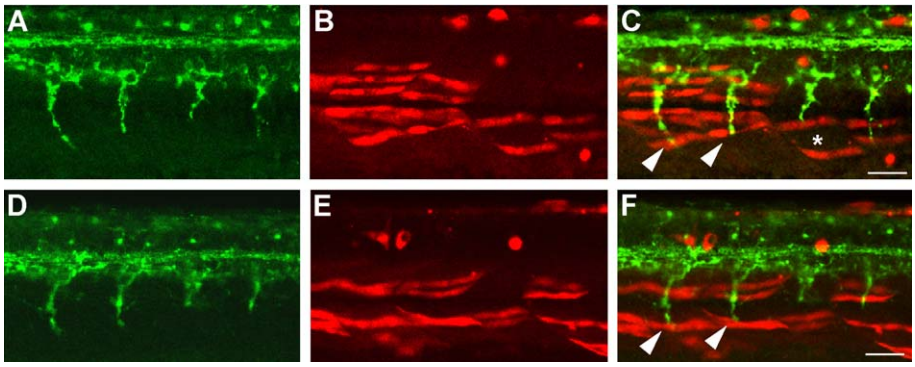


Fig. 7. Wild-type muscle rescues the CaP axon defect in *topped* mutants. Wild-type rhodamine-labeled cells were transplanted into *topped* mutant host embryos. (A and D) Lateral view of a znp1-labeled 27 hpf *topped* mutant embryo containing transplanted wild-type rhodamine-labeled muscle cells (B and E). (C and F) Merged image indicating that the transplanted wild-type muscle is able to rescue the CaP axon defect in *topped* mutants. Arrowheads indicate that CaP axons are rescued to the location of the most ventral muscle cell; asterisk in C indicates a space the width of three muscle cells does not allow rescue of the CaP axon. Scale bar, 20 μm.

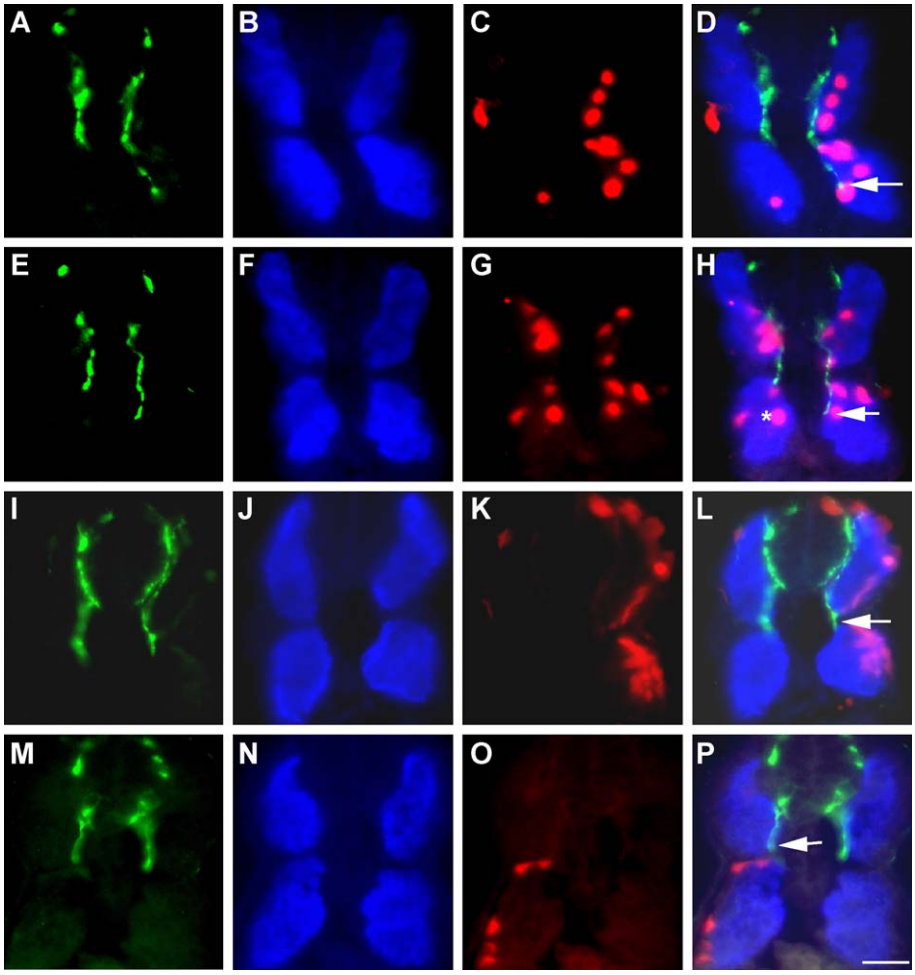
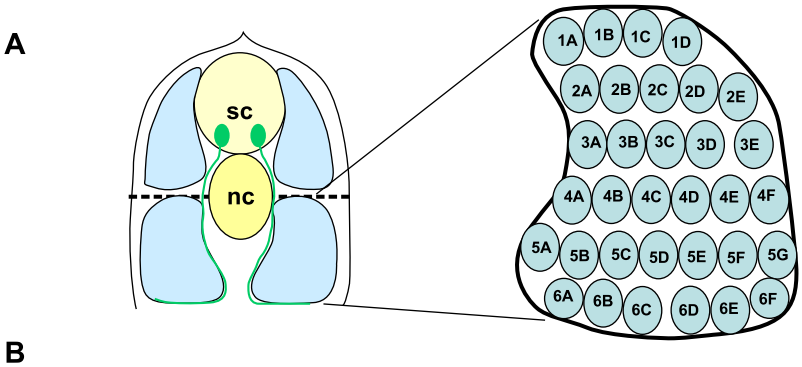


Fig. 8. Topped is functioning in ventromedial fast muscle to promote CaP axon outgrowth into the ventral myotome. Wild-type rhodamine-labeled cells were transplanted into *topped* mutant host embryos. Using cross-sectional analysis, we determined the position of the transplanted muscle (red); (C, G, K, and O) and the corresponding position of F310-labeled fast muscle cells (B, F, J, and N) in *topped* mutants as shown by antibody labeling with znp1 (A, E, I, and M). Merged image of rhodamine, FITC, and DAPI images indicates the juxtaposition of the CaP axon and the transplanted wild-type muscle (D, H, L, and P). Pink cells in (D, H, and L) indicate doubly labeled fast muscle cells. Only ventromedial fast muscle was able to rescue the CaP axon defect in *topped* mutants (A–D) and (E–H). Asterisk in H indicates that a fast muscle cell in roughly the same dorsoventral position as the one in the corresponding hemisegment is not able to rescue the CaP axon defect due to its more lateral position. (I–L) The presence of ventrolateral fast muscle cells or slow muscle cells at the horizontal myoseptum (P) failed to rescue the CaP axon defect in *topped* mutants. White arrows denote CaP axons are rescued to the location of transplanted wild-type fast muscle cells. Scale bar, 30 μm.



B

Position of wild-type	Examples (number of times observed)								
transplanted muscle cells	1 (4)	2 (4)	3 (3)	4 (1)	5 (1)	6 (2)	7 (2)	8 (1)	9 (1)
1A	●	●	●	●	●	●	●		
2A		●				●	●		●
3A			●			●	●	●	
4A				●	●		●	●	
5A					●				

Fig. 9. Schematic depiction of wild-type muscle cells that rescue CaP axons in *topped* mutants. (A) Cross section of the mid trunk of a 26-hpf embryo. Fast muscle is indicated in blue and CaPs in green. For clarity, CaP cell bodies and axons are the only primary motoneurons shown. Each fast muscle cell within the myotome is given a number and letter designation. These designations are indicative of position within the myotome and are not meant to imply identified muscle fibers. (B) Table indicating the position of rescuing wild-type fast muscle cells (red) as seen in the genetic mosaics. We observed nine different rescues schemes and in each example CaP axons were rescued to the ventral-most wild-type muscle cell. sc, spinal cord; nc, notochord.

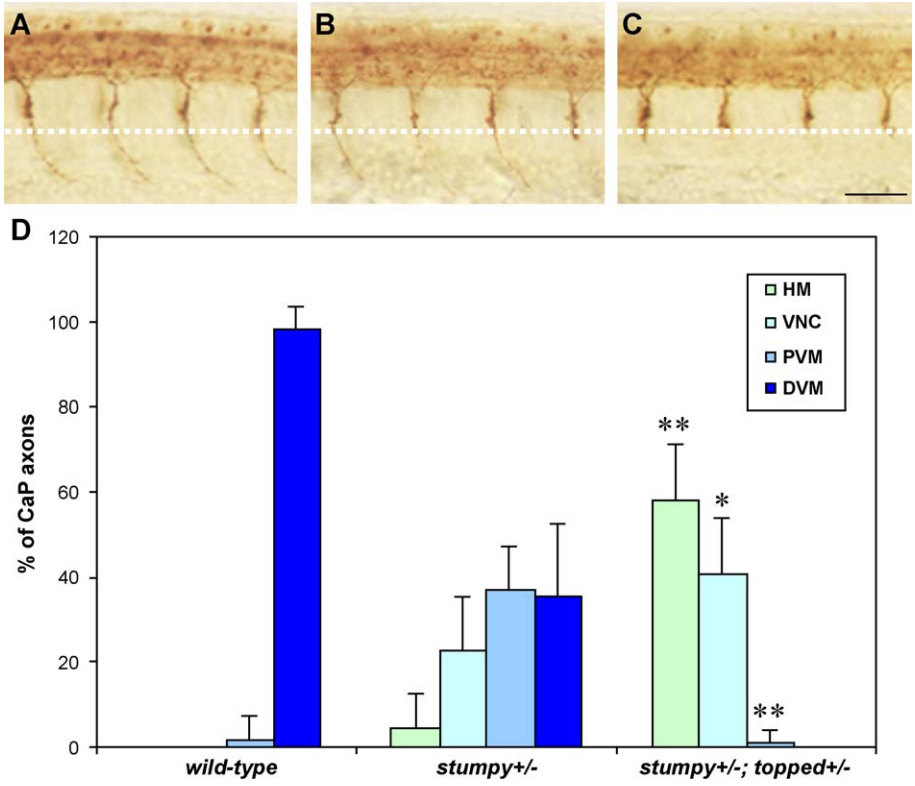


Fig. 10. *topped* and *stumpy* genetically interact. (A) Lateral view of a 26-hpf wild-type embryo. *stumpy*$^{393-/-}$ fish were mated with *topped*$^{+/-}$ fish to generate *stumpy*$^{b393+/-}$;*topped*$^{+/-}$ transheterozygotes (C) and *stumpy*$^{b393+/-}$ siblings (B). White dashed line denotes the first intermediate target. (D) CaP axon position was counted in hemisegments 8–12; n = 110 CaP axons in 11 embryos for wild-type, *stumpy*$^{393+/-}$, and *stumpy*$^{393+/-}$;*topped*$^{+/-}$ embryos. Axon positions were scored as HM (horizontal myoseptum), VNC (ventral edge of notochord), and VM (ventral muscle) and plotted as mean ± 95% confidence interval. The statistical difference in growth cone position between *stumpy*$^{393+/-}$ and *stumpy*$^{393+/-}$; *topped*$^{+/-}$ embryos was determined by Student's t test with *P = 0.01–0.001; **P < 0.001. Scale bar, 35 μm.

Table 4). Additionally, slow muscle was also unable to rescue the axon defect (8 M–P; Table 4). Even the presence of three slow muscle cells at the first intermediate target did not rescue mutant CaP axons (Figs. 8M–P). Taken together, these results indicate that Topped is functioning over short distances very specifically in ventromedial fast muscle to enable CaP axon outgrowth into the ventral myotome.

topped and stumpy function in the same genetic pathway

To examine whether *topped* interacted with other genes involved in CaP axon guidance, we generated transheterozygotes with *stumpy*. In *stumpy* mutants, motor axons stall at intermediate targets and fail to extend into distal myotome regions (Beattie et al., 2000). *topped* heterozygotes do not have a phenotype and $stumpy^{b393}$ heterozygotes display a mild-stall phenotype (Beattie et al., 2000; Fig. 10B). If *topped* and *stumpy* were in the same genetic pathway, then we would predict that lacking one copy of both *stumpy* and *topped* would cause a more pronounced stall phenotype at the first intermediate target than that seen in $stumpy^{b393}$ heterozygotes. Using antibody labeling, we found that $stumpy^{b393+/-}$;$topped^{+/-}$ embryos had a more severe stall phenotype at the first intermediate target than $stumpy^{b393}$ heterozygous embryos (Fig. 10). There was a 92% increase in the number of CaP axons stalled at the first intermediate target in the transheterozygotes versus *stumpy* heterozygotes at 26 hpf. By 48 hpf, CaP axons in the double heterozygotes had recovered suggesting that the transheterozygous phenotype is less severe than the *stumpy* homozygous phenotype (see Beattie et al., 2000). Therefore, partially eliminating *topped* can worsen the CaP stall phenotype in *stumpy* heterozygotes. The same interaction was observed using a recessive, lethal allele of *stumpy*, $stumpy^{b398}$. To address whether this interaction was unique to *stumpy* and *topped*, we generated transheterozygotes with another mutation that affected CaP axons, *unplugged*. In *unplugged* mutants, CaP and RoP axons fail to undergo appropriate pathfinding at the first intermediate target (Zhang and Granato, 2000). We found no evidence of a genetic interaction in *stumpy;unplugged* (n = 60 embryos from two crosses) or *topped;unplugged* transheterozygotes (n = 120 embryos from three crosses; data not shown). These data support the idea that *topped* and *stumpy* act in the same genetic pathway to promote CaP axon outgrowth into the ventral myotome.

Discussion

In *topped* mutants CaP axons stall at the first intermediate target at the nascent horizontal myoseptum, a region that demarcates the boundary between dorsal and ventral muscle. Genetic mosaic analysis reveals that Topped function is required in ventromedial fast muscle for CaP axons to extend ventrally. Furthermore, the degree of rescue is dependent upon the ventral extent of wild-type muscle cells. Taken together, our analysis suggests that Topped functions either as a short-range or membrane-bound cue in the most medial fast muscle cells to define the ventral motor axon pathway.

Ventral motor axon outgrowth is specifically affected in topped mutants

The only defect found in *topped* mutants is delayed axon outgrowth into the ventral myotome. Because this mutant was found in an immunohistochemistry screen designed to uncover defects in primary motor axons (Beattie et al., 1999), it is not surprising that such a subtle mutation was found. The finding that these axons eventually extend into the ventral myotome suggests that this mutation may not be a null allele; alternatively, there may be redundant cues in the ventral myotome. Both primary and secondary motor axons were delayed entering the ventral myotome in *topped* mutants, suggesting either that the secondary motor axon phenotype is due to defects in primary motor axons (see Pike et al., 1992) or that both populations of axons respond to the same cues. Zeller et al. (2002) showed that both primary and secondary motor axons were affected in *diwanka* mutants and that the aberrant secondary motor axons did not directly follow the primary motor axons, suggesting that both populations were responding to pathfinding cues in the myotome. Moreover, in *topped* mutants, we found that secondary motor axons had extended further ventrally at 30 hpf than primary motor axons; again suggesting that the pathfinding defects between the two populations were independent. Therefore, data from mutant analysis support the idea that both primary and secondary motor axons respond to myotomally derived pathfinding cues.

We found no other defects in *topped* mutant embryos suggesting that Topped specifically functions to promote motor axon outgrowth along the ventral myotome. In particular, we did not find defects in the MiP axons, which project dorsally. In *unplugged* mutants, CaP and RoP motor axons are affected at the horizontal myoseptum; however, MiP axons are unaffected (Zeller and Granato, 1999). These data support the idea that the different primary motoneurons are responding to unique cues that enable the formation of their stereotyped axonal projections.

Fast muscle and ventral axon outgrowth

Like other vertebrates, zebrafish have both fast and slow muscle fibers. The origins of these muscle fibers are unique as are their developmental properties. The majority of cells in the zebrafish somite will give rise to the myotome with only a small region of the ventromedial somite giving rise to sclerotome (Morin-Kensicki and Eisen, 1997). Slow muscle starts as a group of approximately 20 cells adjacent to the notochord called adaxial cells, and fast muscle comprises the remainder of the

myotome. In each muscle hemisegment at about 18 hpf, all but three to six slow muscle cells begin to migrate dorsally and ventrally and then radially through the developing fast muscle and ultimately reside as the most lateral cells in the myotome (Devoto et al., 1996; reviewed in Stickney et al., 2000). The slow muscle cells that fail to migrate are located at the horizontal myoseptum and are called muscle pioneer cells (Devoto et al., 1996; Felsenfield et al., 1991). After slow muscle migration, fast muscle becomes the most medially located muscle type in the myotome. The timing of CaP axon outgrowth coincides with the radial migration of slow muscle cells (Zeller and Granato, 1999) such that CaP growth cones extend along ventromedial fast muscle as it is exposed by the migrating slow muscle.

Analyses of two zebrafish mutants, *diwanka* and *unplugged*, have identified cues important for axon guidance that function in slow muscle (Zeller and Granato, 1999; Zhang and Granato, 2000). Diwanka function is needed in only one to three adaxial cells located between the ventral aspect of the spinal cord and the horizontal myoseptum and is necessary for establishing the common pathway used by all primary motor growth cones (Zeller and Granato, 1999). Unplugged function is required in three or more adaxial cells in the same region and is necessary for CaP and RoP to make the correct pathway choice at the first intermediate target (Zhang and Granato, 2000). Diwanka and Unplugged, therefore, appear to function as slow muscle derived signals that affect axon guidance on the fast muscle.

Our analysis of *topped* mutants reveals that fast muscle cells also contain cues essential for directed growth cone migration. The finding that the extent of CaP axon rescue in the genetic mosaics depended on the dorsoventral location of the transplanted wild-type cells and that gaps of three or more wild-type medial fast muscle cells failed to rescue mutant CaP axons suggest that Topped may function as a contact mediated or short-range attractive cue. We found no evidence of abnormal muscle development suggesting that *topped* does not function in fast muscle maturation (see Supplemental Fig. 2). The finding that MiP axons are unaffected in *topped* mutants suggests that Topped does not function as an inhibitory molecule for non-ventrally extending axons.

Genetic pathways as revealed by mutant analysis

The genetic interaction between *stumpy* and *topped* strongly supports that these two genes function in the same genetic pathway. The data do not, however, discern between a direct and an indirect interaction. Using transheterozygotes to analyze genes involved in axon guidance at the midline in *Drosophila* revealed that *roundabout* (*robo*) and Abelson protein tyrosine kinase (Abl) interact genetically and subsequent biochemical analysis showed that this interaction was direct (Bashaw et al., 2000; Wills et al., 2002). A cyclase-associated protein and regulator of actin structure (capulet) also showed a genetic interaction with *robo*; however, it biochemically interacts with Abl not Robo (Wills et al., 2002). Since *stumpy* is needed in both a CaP cell-autonomous and non-cell-autonomous manner (Beattie et al., 2000), it has been difficult to determine where in the environment *stumpy* function is required. Since *stumpy* and *topped* show a genetic interaction, it is possible that *stumpy* is also required in the ventromedial myotome. Cloning these genes and analysis of their proteins will allow us to build upon this genetic interaction with biochemical data.

topped, together with the previously characterized mutants that affect CaP axon pathfinding, indicates that numerous cues are indispensable for wild-type axon guidance. *diwanka* is needed to set-up the common pathway, *unplugged* is needed for CaP and RoP to make the appropriate choice at the first intermediate target, *stumpy* is needed for axons to proceed past intermediate targets, and *topped* is needed for CaP axons to extend into the ventral myotome. Topped function is unique in that it is the only mutation that specifically affects only one class of motoneurons; those extending into the ventral myotome. Studies underway will identify the molecular nature of *topped* and begin to address its biochemical mechanism of action. Moreover, eventual analysis of *topped* in other species may reveal conserved mechanisms establishing dorsoventral innervation of axial muscle.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.06.007.

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